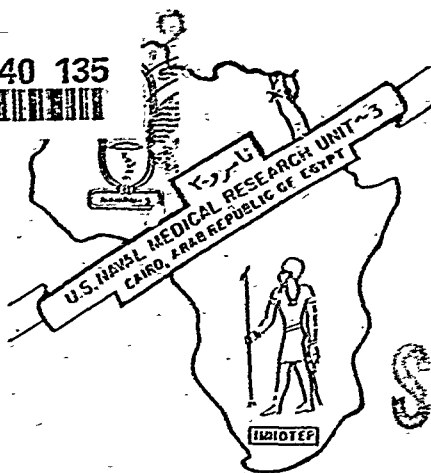


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KEYHOLE LIMPET HEMOCYANIN

BY

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SEROLOGICAL DIFFERENTIATION OF ACUTE AND CHRONIC SCHISTOSOMIASIS MANSONI BY ANTIBODY RESPONSES TO KEYHOLE LIMPET HEMOCYANIN

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Abstract. The existence of a shared epitope between the hemocyanin of the marine mollusk *Megathura crenulata*, better known as the keyhole limpet, and schistosomula has been reported. This epitope has been shown to be a major immunogen in human infection. In this study, keyhole limpet hemocyanin (KLH) was used to measure antibodies recognizing the cross-reacting epitopes in sera from patients with acute and chronic schistosomiasis using an enzyme-linked immunosorbent assay (ELISA). Marked differences in IgG and IgM antibody response were noted between acutely and chronically infected patients at a reciprocal serum dilution of up to 2,560. The acute sera had a mean \pm SD OD_{492 nm} values for IgG and IgM of 1.0 ± 0.44 and 1.34 ± 0.6 compared to mean \pm SD IgG and IgM absorbance for the chronic sera of 0.22 ± 0.10 and 0.22 ± 0.11 respectively. Setting our lowest positive limit at >2 SD above the mean of the chronic sera, 28 of the 30 patients previously diagnosed as having acute schistosomiasis were correctly identified by their IgG and IgM response. Of 5 patients studied longitudinally, IgG persisted at the same levels 10-13 weeks after treatment. IgM levels, on the other hand, showed a tendency to decrease but remained above the established cut-off level. This study provides further evidence for the association of schistosomulum surface carbohydrate antibody with acute infection and demonstrates the ability of a simple non-competitive ELISA using microtiter plates coated with minute quantities of KLH to differentiate serologically between cases of acute and chronic schistosomiasis.

An individual's primary exposure to schistosome infection frequently results in acute, toxic schistosomiasis, a syndrome developing 4-10 weeks after infection and characterized by fever, prostration, diarrhea, and eosinophilia.¹ These symptoms tend to occur in the early stages of the infection, when parasite burdens are low and stool examinations to detect ova may be unreliable. These symptoms generally subside as the infection becomes chronic, even without specific treatment. Little progress has been made in the development of early serological screening for acute schistosomiasis. Numerous antigen preparations have been evaluated and partially successful attempts to distinguish between chronic and acute schistosomiasis on the basis of specific IgM titers and immunoglobulin ratios have been reported.²⁻⁷ All describe tedious and time-consuming methods of preparation for the antigens used in these assays.

Immunoprecipitation and surface binding assays using sera from Egyptian patients infected with *Schistosoma mansoni* have revealed a sig-

nificant difference in the specificities of anti-schistosomulum surface IgG between individuals with acute and chronic infections.⁸ It was found that the $M_r > 200$ kDa antigen, the principal target of carbohydrate antibody on the schistosomulum surface, contributed 40% of the ¹²⁵I-labeled surface molecules precipitated by antibodies from acute patients, but only 13% of the surface molecules precipitated by antibodies from chronic patients. In addition, schistosomulum surface binding assays using pooled sera revealed that 80% of the anti-surface IgG in an acute serum pool was specific for sodium metaperiodate sensitive carbohydrate epitopes. The level of antibody against such epitopes in a chronic serum pool, however, was below the assay detection limits. A possible explanation is that the high level of surface carbohydrate antibody represents an early anti-schistosome response associated with acute infection, and that the level of serum antibody declines dramatically as the infection becomes chronic.

To test this hypothesis, and to investigate the

possibility of using the presence of schistosomulum surface carbohydrate antibody as a diagnostic test for the early detection of acute schistosomiasis, we have undertaken comparisons of acute and chronic schistosomiasis patients by ELISA using hemocyanin of the keyhole limpet (*Megathura crenulata*) as the target antigen. Keyhole limpet hemocyanin (KLH) was selected because it shares a well defined, protective carbohydrate epitope with the surface of *S. mansoni* schistosomula.⁹ The use of this reagent avoids the necessity of purification of specific surface glycoconjugates from the parasite and facilitates a simple assay for specific surface carbohydrate antibody without the need for monoclonal capture or competition. The availability and stability of KLH would allow for the first time the introduction of an extremely simple and cheap diagnostic test for acute schistosomiasis.

MATERIALS AND METHODS

Patients

Sera for this study were obtained from 30 patients with well-defined acute or early schistosomiasis who were previously evaluated at the "fever of unknown origin" ward of the U.S. Naval Medical Research Unit No. 3 in the Abbassa Fever Hospital, Cairo, Egypt. All were town residents who had been exposed briefly for the first time to infected Nile water. Eggs of *S. mansoni* were eventually found in all patients, although repeated stool examinations or rectal snip was required to find the eggs in some cases. The patients were 5-19 years old and had had fevers of 3-8 weeks duration. They also had diarrhea, palpable, tender livers, and eosinophilia of 17-65%. Patients were treated with opraquantel (60-75 mg/kg of body weight divided into 3 doses given in 1 day). Follow-up serum samples were obtained from 5 of these patients 10-13 weeks later.

An additional 30 sera were also obtained from patients with chronic schistosomiasis who had had well-documented active infections for at least 3 years. They were all excreting *S. mansoni* eggs in their stools and were 8-25 years of age.

Since fascioliasis and schistosomiasis commonly coexist in Egypt, sera from 12 patients with proven *Fasciola gigantica* infection but free of schistosomiasis were also included in the study. In addition, sera were obtained from 10 healthy

laboratory investigators to serve as normal controls.

ELISA

A standard micro-ELISA was applied using 50 μ l of each reagent. Polystyrene 96-well, flat-bottom microtiter plates (Linbro, Flow Laboratories, Inc., McLean, VA) were used. Each well was coated overnight at room temperature with 50 ng of KLH (Sigma Chemical Co., St. Louis, MO) in 50 μ l 0.05 M carbonate buffer pH 9.6. This amount of antigen was empirically determined by checkerboard titration using positive and negative sera to be the minimum amount required to yield optimal color formation. Each plate was then washed 3 times with phosphate buffered saline, pH 7.6, containing 0.05% Tween 20 (PBS-Tween). Next, 50 μ l of 12 serial 1:2 dilutions of each serum (beginning with 1:2 dilution) were added and the plates were incubated for 2 hr at room temperature. Serum blanks, antigen blanks, and positive and negative control sera were run with each plate.

The plates were then washed 3 times with PBS-Tween and 50 μ l goat anti-human IgG or IgM peroxidase conjugate diluted 1:2,500 in PBS-Tween (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) were added to each well. After 2 hr, the conjugate was removed and the plates were washed 3 times with PBS-Tween. Each well received 50 μ l peroxidase substrate solution consisting of O-phenylenediamine tablets dissolved in diluent buffer (Abbott Laboratories, North Chicago, IL) immediately before use.

The plates were incubated with substrate for 5-15 min at room temperature and the reaction was stopped with 25 μ l 2.5 M sulfuric acid. The color developed in the plate was read at OD_{490 nm} using an ELISA reader (Titertek Multiskan, Flow Laboratories, Inc., McLean, VA).

Immunoprecipitation

Three hour schistosomula were surface labeled with ¹²⁵I using the Iodogen method.¹⁰ The solubilized surface antigens were immunoprecipitated, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and identified by autoradiography. Rabbit KLH antibody was raised in a sandpiper rabbit by injecting 1m with 100 μ g KLH in complete Freund's adjuvant, boosting 1 week later with a similar



FIGURE 1 Immunoprecipitation of 125 I schistosomulum surface antigens with (a) rabbit KLH antibody and (b) normal rabbit serum. The M_r shown are of the antigens precipitated.

injection, and boosting again 4 weeks later with the same amount of antigen in incomplete Freund's adjuvant. Serum antibody binding to schistosomula surface was done by a competitive binding radioimmunoassay at a serum dilution of 1:10 using 125 I-labeled protein A as described.⁸

RESULTS

Immunoprecipitation of 125 I schistosomulum surface antigens with antibodies raised against KLH in rabbits demonstrated that the antigens with a M_r 17, 38, and >200 kDa express the cross-reactive epitope (Fig. 1). All these antigens have previously been shown to express carbohydrate epitopes.^{11,12} Antigens which have been found to express only non-carbohydrate epitopes such as those with a M_r 32 and 20 kDa were not immunoprecipitated.

TABLE I

Competitive binding of sera pooled from acute and chronic schistosomiasis patients to live schistosomula at 37°C*

Type of serum	Serum alone	Serum + KLH	Serum + egg
Acute	28.2	22.8	4.7
Chronic	4.1	4.4	4.3

* All values are expressed as optical density $\times 10^{-1,000}$ schistosomula and are the averages of triplicates.

To investigate the presence of human antibody to the carbohydrate epitope shared with KLH, sera pooled from Egyptian patients with either acute or chronic schistosomiasis were preabsorbed with KLH and their residual schistosomulum surface binding compared with unabsorbed sera (Table I). Preabsorption with KLH resulted in a 20% reduction in the surface binding of the acute serum pool, but no detectable reduction in the binding of the chronic serum pool. Absorption was also conducted with a schistosome egg homogenate for comparison. This resulted in an 80% reduction in the binding of the acute pool but, again, no reduction in the binding of the chronic pool. These results are consistent with the epitope shared by the schistosomulum surface and KLH representing 25% of the egg cross-reactive carbohydrate epitopes on the schistosomulum surface that are recognized by the acute serum pool.

Preliminary experiments were conducted to examine the possibility of using the ELISA technique to detect antibodies to KLH in the sera of schistosomiasis patients and control subjects. Figure 2 summarizes the results of initial experiments in which pooled sera from patients with acute or chronic schistosomiasis, patients with fascioliasis, and healthy subjects were tested by the ELISA method for the presence of IgG and IgM antibodies to KLH. At all dilutions above 1:40, the mean absorbance at 490 nm of the acute schistosomiasis group was much higher than the mean absorbance of the other 3 groups. Since the titer of antibody against KLH in the acute serum pool was found to be extremely high (>1:20,400) a serum dilution of 1:2,560 was then taken as the titer at which absorbance values at 490 nm were compared in all subsequent experiments.

By the criteria given above, ELISA absorbance obtained from individual sera of patients in the different groups were compared for their IgG and IgM titers as presented in Figure 3. With the

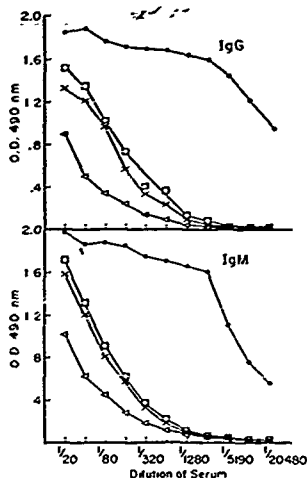


FIGURE 2. Measurement of antibody titer in pooled sera from patients with acute schistosomiasis (●), chronic schistosomiasis (○), fascioliasis (x) and normal controls (Δ) to KLH as measured by ELISA at serum dilution of 1/2,560.

exception of 2 patients in the acute schistosomiasis group and 1 in the fascioliasis group, there was complete separation in both IgG and IgM titers between patients in the acute schistosomiasis group and the other 3 groups. IgM and IgG titers of the chronic schistosomiasis patients was similar to the fascioliasis patients and both were higher than the healthy controls.

The statistical analyses of these data are presented in Table 2. Significant differences were found between the mean ELISA absorbance for the IgG and IgM of the acute schistosomiasis group, the other 2 patient groups, and the healthy control group, but not between the chronic schistosomiasis group and the fascioliasis group. These last 2 groups, however, had significantly higher mean ELISA absorbance readings than the healthy control group at $P < 0.001$, with the exception of the IgG of the fascioliasis group, which was significant only at $P < 0.01$. The data were used to investigate the potential of the anti-

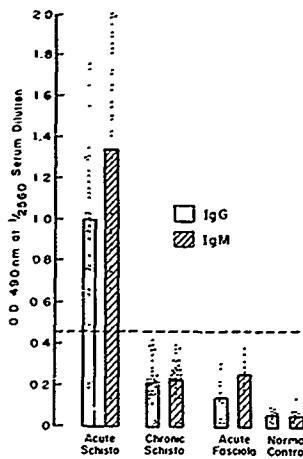


FIGURE 3. Individual and mean absorbance at 490 nm generated by ELISA using sera from patients with acute schistosomiasis, chronic schistosomiasis, fascioliasis, and normal controls.

TABLE 2

Statistical analysis of the IgG and IgM absorbance in sera of patients with acute and chronic schistosomiasis and patients with acute fascioliasis

Type of serum	IgG	IgM
A. Acute Schistosomiasis		
Mean \pm SD	1.0 \pm 0.44	1.34 \pm 0.6
Range	0.18-1.76	0.1-1.98
B. Chronic Schistosomiasis		
Mean \pm SD	0.22 \pm 0.10	0.22 \pm 0.11
Range	0.02-0.39	0.05-0.39
C. Fascioliasis		
Mean \pm SD	0.14 \pm 0.10	0.25 \pm 0.10
Range	0.01-0.30	0.09-0.50
D. Normal Control		
Mean \pm SD	0.05 \pm 0.02	0.05 \pm 0.04
Range	0.015-0.09	0.013-0.124
P A \times B	<0.001	<0.001
P A \times C	<0.001	<0.001
P A \times D	<0.001	<0.001
P B \times C	NS*	NS*
P B \times D	<0.001	<0.001
P C \times D	<0.01	<0.001

*Not significant.

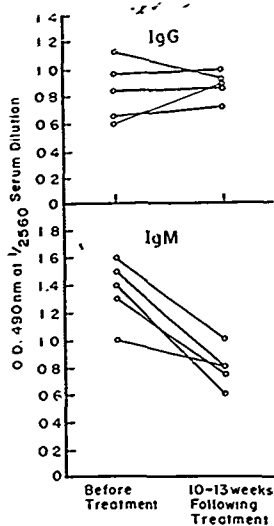


FIGURE 4. Serum anti-KLH IgM and IgG responses in 5 patients with acute schistosomiasis on presentation and 10-13 weeks later, after clearance of the parasite.

KLH ELISA as a diagnostic kit for acute schistosomiasis. Setting our lowest positive limit at slightly >2 SD above the mean of the chronic schistosomiasis group, we established the minimum absorbance value required for a positive acute schistosomiasis diagnosis (lowest positive limit). An OD_{490nm} > 0.45 was then used as the endpoint for the titer determination at serum dilution of 1:2,560.

Given these 2 criteria, 28/30 were correctly scored as positive acute schistosomiasis cases and 2 were false negative by both IgG and IgM titers. No false positive values were detected in the chronic schistosomiasis group. There was 1 false positive value in the fascioliasis group by the IgM response, but not by the IgG response. All control sera from the healthy subjects who were never exposed to infection were correctly identified as negative.

Figure 4 shows serum anti-KLH IgM and IgG responses in 5 patients with acute schistosomiasis for whom follow-up samples were obtained 10-13 weeks following clearance of the parasite. IgM OD readings in all 5 patients showed a definite tendency to decrease, although all values remained above the cutoff value of positivity. On the other hand, the IgG response of these patients showed practically no change at follow-up.

DISCUSSION

Immunization of naive LOU rats with purified KLH has been reported to elicit the production of specific *S. mansoni* antibodies exhibiting both in vitro and in vivo effector functions towards *S. mansoni* schistosomula.⁹ It was further demonstrated that sera collected from KLH immunized rats contain antibodies able to immunoprecipitate from a schistosomula extract a 38 000 M_r surface antigen glycoprotein. This antigen was previously reported to be a major immunogen in human infection, and induced production of specific antibodies in 97% of *S. mansoni* infected patients.¹³

The experimental data fully support the existence of a shared epitope between KLH and schistosomulum surface antigens and demonstrate the presence of the shared epitope on the M_r 17 and >200 kDa antigens, as well as on the M_r 38 kDa antigen as previously reported.⁹ Antibodies from an acute serum pool were shown to bind in significantly higher amounts than those from a chronic serum pool to the surface of schistosomula. KLH competitively reduced the acute serum binding by 20%, but had no effect on the chronic serum binding. This indicates that at least 1 of the epitopes recognized by acute serum on the schistosomulum surface is shared with KLH and that there is insufficient antibody to such epitopes in the chronic serum to allow their detection by this assay.

Based on this finding, experiments were undertaken to quantitate more precisely the level of KLH antibodies in individual sera from patients with schistosomiasis, and to examine the potential use of KLH for the development of an immunoassay that can detect patients with acute *S. mansoni* infection. Initial experiments with pooled sera clearly demonstrated that all types of sera, including that from normal subjects apparently had KLH antibodies at low serum di-

lutions. This titer dropped sharply in all the pooled sera tested except that constructed from acute schistosomiasis patients, where high concentrations of KLH antibodies persisted even at a serum dilution of 1:20,400. This was true whether IgG or IgM antibodies were assayed.

When individual serum assays were performed at a dilution of $\approx 1:2,500$, a highly significant difference between the levels of KLH antibody in the sera of patients with acute schistosomiasis and patients with chronic schistosoma infection was found. This finding fully supports the results of immunoprecipitation experiments which demonstrated that the M_r >200 kDa schistosomulum surface antigen, a major target of schistosomulum surface carbohydrate antibodies, is precipitated at a significantly higher level by antibodies in the sera of acute patients than by antibodies from chronic patients.⁸ Thus the available evidence suggests that schistosome surface carbohydrate antibody is an early response to schistosome infection that declines with time.

It has been shown by others that resistance to reinfection with *S. mansoni* is age-related, apparently developing after some years of infection.¹⁴ Thus it is unlikely that the patients diagnosed as being acutely infected are resistant, although there may be resistant individuals among the patients with chronic infection included in this study, all of whom had a documented history of infection of at least 3 years. Thus it is reasonable to speculate that although carbohydrate antibodies can mediate protective immunity in rodent models,¹⁵ they may not make a significant contribution to resistance to reinfection in man. Indeed it has been proposed on the basis of *in vitro* studies and the analysis of antibody levels in Kenyan children, that schistosomulum surface carbohydrate antibodies may block other protective responses.¹⁶ The work presented here does not contradict this hypothesis.

The difference in KLH antibody levels in the sera of patients with acute and chronic schistosomiasis suggests the use of this glycoconjugate in a specific immunodiagnostic test for acute schistosomiasis. Taking OD_{490 nm} of 0.45 as the cut-off point of positivity, all but 2 subjects in the acute schistosomiasis group were correctly identified, leaving only 2 false negative cases. It is interesting to note that these 2 individuals, though they could not be distinguished clinically from the other acutely infected patients, were the

only individuals in this group to have an IgG/IgM ratio >1 using a soluble egg antigen (data not shown). All of the patients in the chronic schistosomiasis group were correctly detected, and only 1 of the fascioliasis patients showed some cross-reactivity. These results clearly demonstrate for the first time that differential antibody responses to KLH serologically differentiated between acute and chronic schistosome infections.

Serological differences between acute and chronic schistosomiasis were reported by Nash and others.^{17,18} They found specific IgM and IgG antibody to a polysaccharide from the schistosome gut to be highest in patients with acute schistosomiasis and lowest in chronically infected patients. These studies further demonstrated that acutely infected patients had mostly IgM specific antibodies, whereas chronically exposed patients had mostly IgG antibodies to a schistosome specific carbohydrate-containing antigen.¹⁸ In our study, IgG and IgM responses to KLH were comparable in all groups tested. No distinction could be made among the groups based on anti-KLH immunoglobulin ratio (IgG/IgM), as reported in other studies using different antigens.^{3,7}

Of the 30 patients in the acute schistosomiasis group, only 5 gave follow-up sera 10–13 weeks after treatment. Analysis of the antibody titer of these sera revealed practically no change in the IgG response, IgM specific antibodies showed a tendency to drop, but remained above the control cut-off level. Obviously, more follow-up testing would be needed before it can be determined whether this test can be used to measure the efficacy of a specific drug treatment.

One major obstacle to the development of a suitable, simple immunodiagnostic procedure for the detection of patients with acute *S. mansoni* infection has been the lack of a specific antigen which possesses both serological specificity (to be recognized in patients in the early stages of the disease) and sufficient sensitivity (to differentiate between chronic and acute infections). The results of this study suggest that purified KLH preparation in an ELISA fulfills this criteria. KLH is biologically active, being an antigen that sensitizes animals for the production of *S. mansoni* antibodies with similar effector function to those induced during the course of infection. Thus the demonstration of circulating antibodies to this antigen is probably relevant to the presence of an *S. mansoni* infection. It is

commercially available in pure form, precluding the need for lengthy and tedious procedures of isolation, purification, and standardization of antigens. It is cheap and relatively stable in lyophilized form, and as such would be easy to use under field conditions.

This study confirms that high levels of schistosomulum surface carbohydrate antibody are associated with acute schistosomiasis. It demonstrates that quantitative determination of IgG and IgM to *S. mansoni* schistosomulum carbohydrate epitopes, as measured using KLH, can be used as a diagnostic test for the early detection of the disease. Obviously, extensive field testing would be needed to further substantiate the serological specificity of the test.

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19. treatment. IgM levels, on the other hand, showed a tendency to decrease but remained above the established cut-off level. This study provides further evidence for the association of schistosomulum surface carbohydrate antibody with acute infection and demonstrates the ability of simple non-competitive ELISA using microtiter plates coated with minute quantities of KLH to differentiate serologically between cases of acute and chronic schistosomiasis.

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